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Expression of Bcl-2 by Human Bone Marrow Mast Cells and its Overexpression in Mast Cell Leukemia

Carlos Cerveró,[1] Luis Escribano,[1] Jesús F. San Miguel,[2] Beatriz Díaz-Agustín,[1] Pilar Bravo,[1] Jesús Villarrubia,[1] Ramón García-Sanz,[2] José L. Velasco,[1] Pilar Herrera,[1] Mario Vargas,[2] Marcos González,[2] José L. Navarro,[1] and Alberto Orfao[3]*

[1]Servicio de Hematología, Hospital Ramón y Cajal, Madrid, Spain
[2]Servicio de Hematología, Hospital Clínico Universitario de Salamanca, Salamanca, Spain
[3]Servicio de Citometría, Hospital Clínico Universitario de Salamanca, Salamanca, Spain

Bcl-2 protein plays a major role in the prevention of programmed cell death of differentiating cells. In the present study, the expression of cytoplasmic bcl-2 by human Bone Marrow Mast Cells (BMMC) from both normal and pathological bone marrow samples was examined. A total of 35 subjects corresponding to 9 healthy volunteers, 8 cases of adult indolent systemic mast cell disease (SMCD), 4 cases of pediatric mastocytosis (PM), 11 cases of hematological malignancies (HM), 2 cases of reactive bone marrow, and 1 case of mast cell leukemia (MCL) were analyzed. The expression of bcl-2 was studied using quantitative three-color flow cytometry. We also studied the molecular configuration of the bcl-2 gene and other relatives by Southern blot and polymerase chain reaction (PCR) in the MCL case. Bcl-2 expression was detected in BMMC from all samples analyzed. No significant differences on the expression of bcl-2 were detected between BMMC from healthy subjects and patients with SMCD, PM, HM, and reactive bone marrow. By contrast, bcl-2 protein was overexpressed in BMMC from MCL patient without gene rearrangement. Our results show that bcl-2 protein was constitutively expressed by BMMC. BMMC from MCL display overexpression of bcl-2, which could not be related to molecular rearrangements involving the bcl-2 gene. The expression of this protein by mature MC may play a role in the prevention of MC apoptosis and thus help to explain the long survival of these cells. The overexpression of bcl-2 by BMMC in MCL may help to explain their resistance to chemotherapy-induced apoptosis. Am. J. Hematol. 60:191–195, 1999.

Key words: systemic mast cell disease; immunophenotype; flow cytometry; mast cells; bcl-2

INTRODUCTION

Normal tissue homeostasis requires constant regulation of cell growth and cell death. Under physiological conditions, cell death occurs by apoptosis, an active inherently programmed process [1]. The proto-oncogene bcl-2, and recently identified relatives, are the best known intracellular suppressors of apoptosis [2–7]. The bcl-2 proto-oncogene encodes the bcl-2 protein, which is present in mitochondria, endoplasmic reticulum, and perinuclear membranes [3,8]. Lack of apoptosis has been linked to prolonged survival of malignant B-cells expressing the bcl-2 gene [2,3]. Overexpression of the bcl-2 protein have been found in follicular B-cell non-Hodgkin's lymphoma [9], in which molecular evidence of bcl-2 gene rearrangement together with t(14,18) has been demonstrated [9–11]. Overexpression of the bcl-2 protein has also been found in other hematological malignancies (HM). Accordingly, about 10% of B-cell chronic lymphocytic leukemia (CLL) patients display

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*Correspondence to: Dr. Alberto Orfao, Servicio Central de Citometría, Hospital Clínico Universitario, Paseo de San Vicente s/n, 37007 Salamanca, Spain.

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bcl-2 gene rearrangements [12,13], although recent data suggest that overexpression of the bcl-2 protein in CLL is independent of bcl-2 rearrangements [14]. Increased expression of bcl-2 has been also reported in acute lymphoblastic leukemia [15] and multiple myeloma [16], without molecular evidence of bcl-2 gene rearrangement, suggesting that other mechanisms such as abnormalities of the p53 gene may induce overexpression of the bcl-2 protein in these patients. The role of bcl-2 protein in hematopoiesis (revised in reference [17]) has been established in the prevention of programmed cell death of differentiating cells. All hematopoietic lineages that derive from a renewing stem cell express this gene product [18]. Thus, bone marrow precursors of all hematopoietic populations are positive for bcl-2, whereas their mature progeny is usually negative [17].

Mast cells (MC) are long-living cells derived from precursors which originate in the bone marrow [19–22]. MC have been grown ''in vitro'' from CD34+ precursors from bone marrow, cord blood, and peripheral blood mononuclear cells [21–25], and its committed precursor has recently been identified in the mouse [26]. However, until now, no studies have been reported in which bcl-2 expression in human bone marrow mast cells (BMMC) is analyzed.

The aim of the present study was to explore the quantitative expression of bcl-2 protein by BMMC, using three-color flow cytometry (FCM), both in normal controls and in patients with different disease conditions, including mastocytosis.

MATERIAL AND METHODS

Bone Marrow (BM) Samples

BM samples were obtained after informed consent from a total of 35 subjects, including the following: 9 healthy volunteers undergoing either orthopedic surgery or BM harvest, 8 cases corresponding to adult indolent systemic mast cell disease (SMCD), 4 patients diagnosed with pediatric mastocytosis (PM), 1 case of aggressive mast cell disease evolving to a mast cell leukemia (MCL), whose clinical and immunophenotypical characteristics have been described previously [27,28] (this patient was studied at diagnosis and two, five, and ten months after the onset of alpha 2-b interferon (IFN) therapy and later at relapse), 11 patients with HM [2 cases of B-cell CLL, 2 B-cell non-Hodgkin's lymphoma (B-NHL), 2 monoclonal gammopathies of undetermined significance (MGUS), 3 multiple myeloma (MM), and 2 Waldenström's macroglobulinemia (WM)], and 2 cases of reactive BM. The diagnosis of the different types of mastocytosis was made following the recommendations of the consensus classification [29,30].

Immunological Markers Analysis

Samples were collected in EDTA anticoagulant and immediately diluted 1:1 (vol/vol) in phosphate buffered saline (PBS). After collection, BM samples were passed several times through a 25 μm-gauge needle to disgregate the bone marrow particles. Peripheral blood (PB) samples were collected from the patient who had aggressive SMCD at diagnosis, and later at two and five months after IFN therapy, and at relapse.

BM or PB samples were analyzed by direct immunofluorescence using a triple-staining combination of monoclonal antibodies (MoAbs) directly conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), and PerCP. The following antigens were explored: anti-bcl-2-FITC (purchased from Dakopatts, Glostrup, Denmark), CD117-PE (purchased from Nichirei, Tokyo, Japan), and CD45-PerCP (purchased from Becton-Dickinson, San Jose, CA). Briefly, 200 μl of whole PB or PBS-diluted whole BM containing between 0.5 and 1.5 × 10^6 nucleated cells was incubated for 10 min at room temperature with each of the anti-CD117-PE and anti-CD45-PerCP MoAb. Afterward, staining of the intracellular bcl-2 protein was performed using the Fix & Perm reagent (Caltag Laboratories, San Francisco, CA). For that purpose, 100 μl of fixation medium (solution A) were added to the CD117/CD45 stained PB or BM and incubated for 15 min at room temperature; then, cells were washed in PBS by centrifugation for 5 min at 540*g*, and the cell pellet was resuspended in 100 μl of the permeabilization medium (solution B) plus 10 μl of anti-bcl-2 MoAB and incubated for another 15 min at room temperature. Once this incubation was finished, the cells were washed with PBS and resuspended in 500 μl of PBS.

Data acquisition was performed on a FACScan flow cytometer (Becton-Dickinson) using the LYSYS II software program (Becton-Dickinson) for at least 10.000 events/tube. In addition, a second acquisition step, through a SSC/CD117 live gate, was performed to increase the sensitivity of the method for the analysis of MC present at a low frequency [31]. The PAINT-A-GATE plus software program (Becton-Dickinson) was used for data analysis. The quantitation of positivity for bcl-2 was performed using the QuickCal beads (Flow Cytometry Standars Corporation, San Juan, PR) and results expressed as the mean number of molecules equivalent of soluble fluorochrome (MESF) obtained specifically for MC.

DNA Preparation and Southern Blot (SB) Analysis

High molecular weight DNA was isolated from a BM sample of a patient with MCL by standard proteinase K digestion, phenol-chloroform extraction, and ethanol pre-

cipitation. Southern blot analysis was performed after digestion with the Eco RI, Bam HI, Hind III, Bgl II, and Xba I restriction enzymes, size fractionated, transferred and hybridized with ^{32}P-labeled probes as described previously [32]. TcR-β, IgH, Bcl-1, Bcl-2, Bcl-6, c-myc, MLL, p53, and p16 genes were studied using this methodology with the following probes: Cβ1 [33], JH6, MTC, PFL1, PFL2, pB16, Bc16, cD1A, 11q23/MLL, cDNA/p16, and cDNA/p53 [32], respectively.

Polymerase Chain Reaction (PCR) to Detect the t(11,14) and t(14,18) Translocations

The amplification of the breakpoint regions of both translocations was performed by Nested-PCR according to previously described methods [32]. PCR products from the final reactions were analyzed by electrophoresis in agarose and visualized by staining with ethidium bromide [32]. In all experiments a negative control (sterile distilled water and normal DNA) and a positive control (genomic DNA from a positive patient) were used.

Statistical Methods

Mean values and their standard deviations were calculated for all variables in each group of samples. The Kruskall-Wallis and Mann-Whitney U tests were used to assess the statistical significance of the differences observed between groups of individuals.

RESULTS

Enumeration of MC by Flow Cytometry

In all BM samples analyzed, MC were clearly identified based on their strong CD117 expression despite their low frequency. The mean BMMC number for the entire population, excluding the MCL case, was 0.046% (range: 0.0044–4.66%) (Table I).

The number of MC in the MCL case was strikingly high at diagnosis: BM, 40% and PB, 1,5%. Sequential studies showed a decrease in the number of both BM and PB MC at month +2 (BM MC: 25%, PB MC: 0.4%) and +5 (BM MC: 9% and PB MC: 0.1%). Despite maintenance of INF alpha-2b therapy, the number of MC in this patient showed an increase at month +10 (BM MC: 69%; PB MC: 2.3%).

Bcl-2 Expression Determined by Flow Cytometry

Bcl-2 expression was detected in MC from all samples analyzed (Table I and Fig. 1). Interestingly, no significant differences on the expression of bcl-2 were detected between BMMC from healthy subjects ($17.6 \pm 5.8 \times 10^3$ MESF) and patients with SMCD ($18.7 \pm 4.3 \times 10^3$ MESF), PM ($16 \pm 9.9 \times 10^3$ MESF), HM ($21.5 \pm 5.3 \times 10^3$ MESF), and reactive BM ($22.3 \pm 6.4 \times 10^3$ MESF).

By contrast, in the analysis of the case of MCL, expression of the bcl-2 protein in BMMC was higher (70.3×10^3 MESF) than that observed in the other patient categories and healthy subjects and did not vary during the clinical evolution (69.7×10^3 MESF at month +2 of INF-prednisone therapy and 64.7×10^3 MESF at relapse).

Interestingly, as compared with BMMC from normal healthy subjects, PBMC from this patient also displayed increased levels of bcl-2 protein although its expression was lower than that detected in the BMMC from the same patient (34.7×10^3 MESF and 35.6×10^3 MESF at

TABLE I. Distribution of BMMC and bcl-2 Expression as Assessed by Flow Cytometry*

Diagnosis	Bone marrow MC (%)	bcl-2 expression (MESF × 10^3)
Healthy controls (n = 9)	0.026 ± 0.0264	17.6 ± 5.8
SMCD (n = 8)	0.261 ± 0.164	18.7 ± 4.3
PM (n = 4)	1.264 ± 2.264	16 ± 9.9
HM (n = 11)	0.088 ± 0.156	21.5 ± 5.3
RBM (n = 2)	0.617 ± 0.612	22.3 ± 6.4
MCL[a] (n = 1)	40	70.3

*SMCD, adult systemic mast cell disease; PM, pediatric mastocytosis; HM, hematological malignancies; RBM, reactive bone marrow; MCL, mast cell leukemia.
[a]Diagnostic sample.

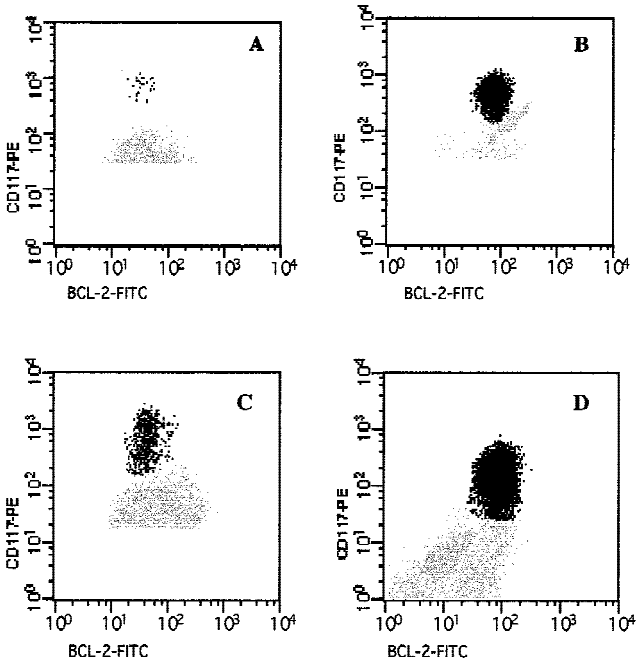

FIG. 1. Representative dot plots of the expression of bcl-2 by BMMC (black dots) from a normal control (A), a case of PM (B), a patient with indolent SMCD (C), and an MCL (D), once selected on the basis of a high expression of CD117. The MESF values (×10^3) are as follows: A, 11.7; B, 18.4; C, 20.7; D, 70.3.

month +2 of INF-prednisone therapy and at relapse, respectively).

Molecular Analysis

All genes analyzed in the BM from the MCL patient (TcR-β, IgH, Bcl-1, Bcl-2, Bcl-6, c-myc, MLL, p53, and p16) showed a germline configuration. In addition, no amplification of the t(11;14) and t(14;18) breakpoint regions were observed by PCR.

DISCUSSION

In the present study, the expression of bcl-2 protein by human MC was evaluated extensively using quantitative flow cytometry. Despite their low frequency, and the changes in their light scatter characteristics induced by cellular permeabilization, MC were easily identified in all samples analyzed using the technical approach described previously [31].

Bcl-2 was constantly detected in BMMC both from normal controls and patients with different disease conditions including mastocytosis. To the best of our knowledge, this is the first report in which bcl-2 expression by human MC was analyzed. The level of bcl-2 expression was similar in BMMC from normal controls, and patients with HM, SMCD, and PM. By contrast, bcl-2 overexpression was found both in BM and PB MC from a patient with MCL; this abnormally high expression of bcl-2 remained stable during the remission phase induced by IFN-prednisone therapy and later at relapse.

MC are long-living cells. Accordingly, they need regulatory mechanisms to prevent programmed cell death. This may help to explain the finding of bcl-2 expression in BMMC from all groups of individuals analyzed in this study; additionally, these findings may suggest that the expression of this protein by mature MC may play a role in the prevention of MC apoptosis and thus help to explain the long survival of these cells, together with the ability of SCF to prevent apoptosis in these cells [34] by a bcl-2-independent mechanism [35]. The overexpression of bcl-2 by both BMMC and PBMC in MCL may play a crucial role in their resistance to chemotherapy-induced apoptosis [36].

Molecular analysis did not detect a rearrangement of the bcl-2 gene that could contribute to an explanation of its overexpression in MC from the MCL patient; in line with these findings, conventional cytogenetics did not show any abnormality involving chromosome 18 (data not shown). Interestingly, the expression of bcl-2 did not vary during IFN alpha-2b therapy despite the clinical, biological, and cytological response, suggesting that the IFN-induced response in mastocytosis was not related to a down-regulation of bcl-2 expression. As a matter of fact, the mechanism of IFN alpha-induced response in MC disease remains unknown, despite its ability to reduce both the spontaneous and induced release of histamine by MC in vitro [37].

In summary our results show that both normal and reactive BMMC display relatively high levels of bcl-2 expression, no significant differences being detected between them. By contrast, MC from MCL display overexpression of bcl-2 which could not be related to molecular rearrangements involving the bcl-2 gene.

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